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# STUDIES ON THE CULTIVATION OF THE VIRUS OF VACCINIA, III

WITH A NOTE ON THE GLYCERIN RESISTANCE OF VARIOUS  
ORGANISMS \*

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In previous work on the virus of vaccinia, done in conjunction with Drs. Lambert and Israeli,<sup>1</sup> it was found that, in combination with tissue cultures, there was a definite multiplication of the virus with active transfers to the third generation and a resistance of the virus to thirty-four days' incubation at 37 C. We found that living tissue was necessary to the growth of the virus in this method and that the cornea of the rabbit or guinea-pig was more favorable than the heart, liver, or kidney. In the corneal preparations, the epithelium showed an active lateral spreading throughout the clot, forming sheets or groups of cells in the plasma. The cells early showed an accumulation of fat in their cytoplasm, but frequently retained their form for several weeks, even when not transferred to fresh plasma. In these preparations, altho the corneal cells were living and the virus of vaccinia was multiplying, we were unable to find any specific vaccine bodies; only smaller, undifferentiated forms have been seen. These undifferentiated forms have been found in the controls in incubated corneal preparations without the vaccine virus and in the incubated virus corneal preparations. We have observed numerous granules in the incubated preparations, but these have not been sufficiently definite in character to allow us, with the methods employed thus far in our studies, to make any positive statements in regard to them.

There is no growth of the virus of vaccinia in preparations containing cornea killed by freezing, or by hypotonic salt solutions, nor in preparations in which pieces of paraffin are substituted for tissue. The virus is soon rendered inactive in preparations containing plasma and cornea from an immune animal, altho the corneal epithelium may grow very well.

\* Received for publication December 3, 1914.

1. Jour. Infect. Dis., 1913, 13, p. 294; Ibid., 1914, 14, p. 87.

Having thus shown multiplication of the virus of vaccinia outside of the body, altho only in conjunction with living tissue preparations, we wish to record at this time results of the purification of the virus for cultural experiments, and culture experiments according to the usual and special bacteriological technic, including a repetition of Fornet's methods. We have also continued our experiments on the resistance of organisms to glycerin.

#### EXPERIMENTS

*Purification of the Virus.*—To obtain an active virus, freed from contaminating organisms, several methods have been used. The sterilization with ether, as described by Fornet,<sup>2</sup> was unsuccessful in our hands, altho tested on a number of viruses taken from both calves and rabbits. We were unable to obtain a virus purified by ether that was not almost entirely inactivated, thus agreeing with the results of Gins.<sup>3</sup> The method of sterilization by the use of chloroform vapors, as recommended by Green<sup>4</sup> and by Nijland,<sup>5</sup> gave us somewhat more favorable results, but the virus was frequently greatly weakened.

The method which gave us the best results was the one originally used by us in the tissue culture experiments, namely, dialyzation after purification by carbolic acid and glycerin. If the virus was then not entirely free from contaminating organisms, carbolic and glycerin were again added and the dialysis repeated, giving a virus both active and pure. This dialyzed virus was used largely in our present work, altho the ether and chloroform viruses were also tested.

The activity of the virus was always tested by the method of Calmette and Guerin, which consists in inoculations of the freshly shaven skin of a rabbit.

*Cultural Experiments.*—In experiments on the growth of the virus of vaccinia, Fornet records activity of the virus after numerous transfers on the usual media, agar and broth, kept anaerobically at incubator temperature. The viruses were purified by ether, and the dilution of the original virus by the transfer was very great. We have made numerous attempts with both the calf and the rabbit viruses to repeat Fornet's work, but always with negative results. These negative results agreed with those of Nijland.

2. Berl. klin. Wchnschr., 1913, 50, p. 1864.

3. Ibid., 1914, 51, p. 391.

4. Lancet, 1903, 1, p. 1738.

5. Arch. f. Hyg., 1906, 56, p. 361.

We also have not been able thus far to cultivate the virus of vaccinia by usual or special bacteriological technic. The inoculated media have been kept both aerobically and anaerobically at temperatures varying from 33-37.5 C. The virus was purified usually by one of the three methods given, but we have also used the pure, active, and multiplying virus from incubated virus tissue preparations. The media inoculated were tubes of beef agar, salt free veal agar, glycerin agar, glucose agar, and 1 percent serum (calf or horse) agar; broth, acid, or alkaline with calcium; egg media, Dorset's, solid and non-inspissated; rabbit or guinea-pig plasma and tissue; Noguchi media, with ascitic fluid, horse or calf serum; Hata media; and plates of plain (beef) agar, or in mixture with animal tissues or fluids.

In no instance have we observed any evidence of multiplication of the virus. On solid media the virus remained active longest on agar plates streaked with purified virus; five to eight weeks when kept anaerobically at 33 C. Neither the original agar plate nor transfers to fresh agar plates or to other media gave any indication of growth of the virus. On solid egg and in Noguchi media, the virus has remained active for two to four weeks. The virus rapidly lost its activity when incubated in fluid media containing calcium, or of an alkaline or neutral reaction. In broth, 2-2.8 percent acid to phenolphthalein, the virus remained active a number of weeks and to the second and third transfers. There was however no indication of multiplication, the activity being accounted for by a dilution of the original virus. Similar results were obtained with fluid egg, non-inspissated Dorset's medium, fluid Noguchi, and Hata medium, and also in broth filtered from old tubercle cultures which contained 5 percent glycerin and was 2.8 percent acid.

#### RESISTANCE OF ORGANISMS TO GLYCERIN IN THE COLD

In work done with Dr. Poor on the virus of rabies, we compared the glycerin resistance of that virus with those of the diphtheria and tubercle bacilli. When acted on in the cold by glycerin, we found the diphtheria bacilli were alive after two weeks, while after thirteen months the tubercle bacilli were alive and virulent to guinea-pigs, thus showing a glycerin resistance as great for that organism as for the virus of rabies or vaccinia.

In our present work, we have continued our experiments on a larger series of organisms of a known nature, including the spirochete

of syphilis. The technic in these experiments was similar to that in the former work; vigorous cultures on solid media were covered with 3-5 c.c. of sterile, neutral glycerin and kept in the ice-box. At definite intervals, transfers of the culture were made to fresh media, solid and fluid, carrying over as little as possible of the glycerin. These transfers were then incubated.

TABLE 1  
SHOWING RESISTANCE OF ORGANISMS TO GLYCERIN IN THE COLD

Organism	After 3 days*	After 6 days	After 14 days	After 21 days	After 30 days	After More Than One Month
<i>Proteus vulgaris</i> .....	+	+	—			
<i>B. prodigiosus</i> .....	+	++	+	....	±	One colony after 32 days
<i>Sta. pyogenes aureus</i>	+	++	+			
<i>Pneumococcus</i> .....	—	—	—			
<i>Streptococcus</i> .....	—	—	—			
<i>Meningococcus</i> .....	—	—	—			
<i>B. diphtheriae</i> 8.....	+	+	+	—	—	
<i>B. diphtheriae</i> 17.....	+	+	+	±	—	
<i>B. pyocyaneus</i> .....	—	—				
<i>B. mallei</i> .....	++	++	+	+	±	Growing less vigorously — 5 colonies after 32 days
<i>B. coli communis</i> (1)	++	++	+	+	±	++ after 41 days
<i>B. coli communis</i> (2)	++	++	++	++	++	
<i>B. typhosus</i> .....	+	±	—			
<i>B. dysenteriae Shiga.</i>	—	—				
<i>V. cholerae</i> .....	±	—				
<i>B. tuberculosis</i> 72.....	+	....	—			
<i>B. tuberculosis</i> 305....	±	....	±	....	±	Very feeble growth after 50 days
<i>B. tuberculosis</i> 311....	—	....	—	....	—	
<i>B. tuberculosis</i> 422....	—	....	±	....	±	Feeble growth after 50 days
<i>B. tuberculosis</i> , Courmont	++	+	++	....	....	Vigorous after 50 days
<i>B. tuberculosis</i> , Dr. L.	....	±	±	....	....	Alive after 50 days
<i>B. tuberculosis</i> , Koch	++	....	+	....	....	Alive after 50 days
<i>B. tuberculosis</i> , Ravenel	++	....	±	....	....	Very feeble growth after 50 days
<i>Spirochaeta pallida</i> ..	—	—				

\* ++, vigorous growth; +, moderate growth; ±, feeble growth; —, no growth.

The glycerin resistance of the spirochete was tested as follows: A small piece of a nodule from an inoculated testis of a rabbit, which showed abundant spirochetes, was immersed in glycerin and kept in the ice-box. After seventy-two hours this piece was removed, emulsified, and inoculated into a rabbit's testis with negative results, while inoculations from the original material proved virulent. A culture of spirochetes from a strain received originally from Dr. Noguchi, was subjected to glycerin in the cold. Seventy-two hours later, transplants were made, which did not grow when incubated, while control transfers grew. These results were not surprising, since the

*spirochaeta pallida* is killed by drying. However, not all the organisms which resisted desiccation were glycerin resistant.

In our present work, the most resistant of the bacteria examined were the tubercle bacilli, transfers of which grew after the glycerin had been on the cultures for fifty days (they were not tested further), and then the staphylococcus and the colon bacillus, which were still alive after thirty to forty days. The typhoid bacillus did not resist more than six days. Table 1 shows the work in detail.

#### SUMMARY

Cultural experiments with the virus of vaccinia, with usual or special bacteriological methods, were negative. There was no evidence of a multiplication of the virus. The virus, streaked on agar plates and kept anaerobically, has remained alive for eight weeks at 33 C. An acid reaction of media appears favorable to the virus.

A comparative study of the action in the cold of glycerin on organisms of a known nature shows the resistance to be a variable one, not always paralleled by resistance to drying. Of the organisms tested, the tubercle bacilli were the most resistant, then the staphylococci and colon bacilli.